Journal of Insect Physiology 68 (2014) 1–6

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Methyl eugenol aromatherapy enhances the mating competitiveness of male *Bactrocera carambolae* Drew & Hancock (Diptera: Tephritidae)

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ARTICLE INFO

Article history:
Received 6 November 2013
Received in revised form 6 June 2014
Accepted 18 June 2014
Available online 1 July 2014

Keywords:
Carambola fruit fly
Mating success
Methyl eugenol treatment
Sterile insect technique

ABSTRACT

Males of *Bactrocera carambolae* Drew & Hancock (Diptera: Tephritidae) are strongly attracted to methyl eugenol (ME) (1,2-dimethoxy-4-(2-propenyl)benzene), a natural compound occurring in variety of plant species. ME-feeding is known to enhance male *B. carambolae* mating competitiveness 3 days after feeding. Enhanced male mating competitiveness due to ME-feeding can increase the effectiveness of sterile insect technique (SIT) manifolds. However, the common methods for emergence and holding fruit flies prior to field releases do not allow the inclusion of any ME feeding treatment after fly emergence. Therefore this study was planned to assess the effects of ME-aromatherapy in comparison with ME feeding on male *B. carambolae* mating competitiveness as aromatherapy is pragmatic for fruit flies emergence and holding facilities. Effects of ME application by feeding or by aromatherapy for enhanced mating competitiveness were evaluated 3d after treatments in field cages. ME feeding and ME aromatherapy enhanced male mating competitiveness as compared to untreated males. Males treated with ME either by feeding or by aromatherapy showed similar mating success but mating success was significantly higher than that of untreated males. The results are discussed in the context of application of ME by aromatherapy as a pragmatic approach in a mass-rearing facility and its implications for effectiveness of SIT.

1. Introduction

Methyl eugenol (ME) (1,2-dimethoxy-4-(2-propenyl)benzene), a phenylpropanoid compound found in >450 plant species (Tan and Nishida, 2012), is a powerful attractant for males of many tropical tephritid fruit fly species of the genera *Bactrocera* and *Dacus* (Drew, 1974, 1989; White and Elson-Harris, 1992; Shelly, 2010). This behavioural response has been exploited since the 1950s for population monitoring and as part of an environmentally-friendly, lure-and-kill approach for controlling species of economic importance termed male annihilation technique (MAT) (Barclay et al., 2014; Christenson, 1963; Cunningham and Suda, 1986; Steiner and Lee, 1955). The MAT has been used to suppress *Bactrocera* pest species as part of integrated pest management programmes and even to eradicate isolated populations, such as on islands or during outbreaks: e.g., *Bactrocera dorsalis* Hendel from the Marianas Islands, Micronesia (Steiner et al., 1970), various *Bactrocera* spp. from California (USA) on several occasions (Chambers, 1977; CDFA, 2013), and *B. dorsalis* from the Okinawa Islands, Japan (Koyama et al., 1984). Nevertheless, the MAT failed to eradicate *B. dorsalis* from the Ogasawara Islands of Japan (Christenson, 1963), perhaps owing to the evolution of non-response to ME among wild males (Iwahashi, 1973). The efficacy of the MAT may also be compromised where wild males have access to abundant natural ME sources as consumption of sufficient amounts of the chemical can result in reduced attraction to ME baited traps (Shelly, 1994). Moreover, the MAT needs to be applied in a very systematic way and on an area-wide basis; otherwise, even if a large proportion of the male population has been killed, a few surviving or immigrating males can still fertilize many females, and the economic damage caused by ovipositing females is not significantly reduced (Steiner et al., 1970; Cunningham, 1989). For eradication programmes, therefore, after reducing the population using the MAT, the sterile insect technique (SIT) has been successfully integrated to achieve final eradication (Steiner et al., 1970; Itô and Iwahashi, 1974; Koyama et al., 1984). The

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http://dx.doi.org/10.1016/j.jinsphys.2014.06.014
0022-1910/

SIT, which is likewise an environmentally friendly technique, involves mass-rearing of male insects, sterilizing them by ionizing radiation, and releasing them in the target area in numbers large enough to outcompete their wild counterparts (Knipling, 1955; Dyck et al., 2005a). Wild females that mate with sterile males produce no off-spring, and therefore the release of sterile males in adequate numbers reduces the wild population. In certain cases, this population suppression can lead to eventual eradication of the target population (Hendrichs and Robinson, 2009). Furthermore, as the SIT acts in an inverse density dependent manner, it becomes more effective when the wild population is reduced (Dyck et al., 2005b; Knipling, 1979; Vreysen et al., 2006). Integration of the MAT with the SIT has so far been sequential, rather than simultaneous, with the SIT applied after a significant reduction of the wild population with the MAT (Itô and Iwahashi, 1974; Shiga, 1989; Steiner et al., 1970) to avoid the mass-trapping of the released sterile males in ME-baited traps, which would significantly reduce the efficacy of the SIT.

Nevertheless, there is potential for simultaneously implementing the SIT and the MAT (Chambers et al., 1972; Barclay et al., 2014). Male *B. dorsalis* exposed to ME before being released were two to three times less likely to be captured in ME-baited traps than non-ME exposed males (Shelly, 1994). Sterile males of *B. dorsalis* that have been given access to ME before their release would therefore not respond to ME traps, whereas wild male flies would continue actively to seek out ME sources in nature and would therefore continue being attracted and eliminated at ME baits/traps. This 'male replacement' approach (reducing the wild males at ME traps while simultaneously releasing sterile males) will significantly increase the sterile to wild male over-flooding ratios and reduce the number of sterile males that need to be released (Barclay et al., 2014; McInnis et al., 2011; Robinson and Hendrichs, 2005). In addition, pre-release feeding of ME enhances mating competitiveness of the released males compared to males that have not been fed ME (Shelly and Dewire, 1994). Biochemical assays of ME metabolism have shown that the sex pheromone of ME-Fed males contained metabolites of this compound, whereas volatiles of unfed males lacked these metabolites (Nishida et al., 1988b). Furthermore, behavioural studies also showed that wild males *B. dorsalis* which ingested ME exhibited increased signalling behaviour, signal attractiveness, and mating success compared with males that had not been given access to the lure; females are also more attracted to pheromone blends of ME treated males (Shelly and Dewire, 1994; Tan and Nishida, 1996). The enhanced mating success of sterile males due to ME feeding has implications for the SIT. For example, Shelly et al. (2010a) demonstrated in field enclosure tests that the same level of egg sterility could be induced with 84% fewer ME treated sterile males as compared to ME deprived males.

Bactrocera carambolae Drew & Hancock (Diptera: Tephritidae) is native to Indonesia, Malaysia, and Thailand. Furthermore, in 1975 it was collected in Paramaribo, Suriname, South America, and has since spread to French Guyana and northern Brazil (Godoy, 2006; van Sauers-Müller, 2008). It is a pest of economic importance, causing economic losses to more than 150 fruit species and has been declared a quarantine pest insect in the Caribbean region, interfering with international trade of fruits and vegetables (Malavasi et al., 2000). Like other species included in the *B. dorsalis* complex, *B. carambolae* males are also attracted to ME (Iwahashi et al., 1996). ME is routinely used to monitor populations of *B. carambolae*, and the MAT eradicated some populations in Surinam and Guyana (Malavasi et al., 2000). Behavioural assays of ME consumption showed that ME-Fed males initiated aggregations at dusk, started sexual calling earlier, and achieved higher mating success than non-ME-Fed males (Wee et al., 2007). Furthermore, chemical assays confirmed that *B. carambolae* males metabolized the ME compounds and converted it into coniferyl alcohol (CF); females are more attracted to pheromones containing CF (Wee et al., 2007).

Despite the effect of ME on male mating success across *Bactrocera* species (Shelly et al., 1996; Tan and Nishida, 1996; Orankanok et al., 2013) and its huge potential impact on cost-effectiveness of the SIT, its use at fly emergence and release facilities has been limited by the lack of a suitable delivery system to mass-reared sterile males before their release in the field. The common methods for emergence and holding fruit flies prior to field releases (Mabry, 1986; Salvato et al., 2004; Enkerlin, 2007) do not support the inclusion of any ME feeding treatment after fly emergence. As exposure to ME must be brief in view of its toxicity due to unlimited feeding (Steiner, 1952), Tan and Tan (2013) designed a machine where sterile males can be fed on ME using a ME-impregnated relaying belt, after which males are brushed off and collected. While this is an innovative system for use under experimental conditions, it is not suitable for treating millions of sterile males per day on an industrial scale. Therefore, there is need to develop a simple method of exposing sterile males to ME in emergence and holding facilities that is compatible with current emergence and holding conditions.

The current system being routinely used to increase mating success of Mediterranean fruit fly sterile males (Enkerlin, 2007; Shelly et al., 2010b) consists of exposing the males through aromatherapy to the volatiles of ginger root oil (GRO) in the fly holding rooms. Thus, sexually mature males can inhale or be impregnated with the chemicals from the circulating air prior to their release in the field, thereby improving the SIT component of various operational action programs (Shelly et al., 2007, 2004; Silva et al., 2013). The objective of this study was to assess whether ME aromatherapy enhances mating competiveness of *B. carambolae* males and whether ME aromatherapy can replace ME feeding.

2. Material and methods

2.1. Study insects

The *B. carambolae* flies used in this study originated from Paramaribo, Suriname, and had been cultured for 26 generations at the FAO/IAEA Insect Pest Control Laboratory of the Agriculture and Biotechnology Laboratories in Seibersdorf, Austria. The colony was maintained on a carrot powder based larval diet that was modified from the standard (wheat bran-based) Seibersdorf diet (Hooper, 1987). Following emergence, the flies were provided with standard adult diet (sugar: hydrolyzed yeast; 3:1 by proportion) and supplied with water *ad libitum*. The flies were sexed within 3d after emergence (well before reaching sexual maturity at day 15 post emergence, Haq, unpublished data), transferred to plexiglass tubular cages (45 cm × 64 cm diameter) having both openings covered with cloth mesh, and supplied with standard adult diet and water *ad libitum*. The flies were maintained in the laboratory at 24 ± 1 °C, 60 ± 5% RH and a photoperiod of 14L:10D. The flies used in a given experiment were from the same batch of pupae. Males were marked on the thorax by different colours of a water-based paint before different treatments.

2.2. Treatments

2.2.1. ME-feeding

ME-feeding was conducted in a room isolated from the fly culture room. Males were marked 1d earlier by holding non-anaesthetized individuals motionless in nylon netting and applying water-based paint to the thorax. Marked males (15–16d old, $n = 100$) were transferred to a plexiglass tubular cage

(20 cm × 64 cm diameter) having both openings covered with cloth mesh. ME (0.5 mL) was placed on a filter paper strip, which was then placed in a petri-dish (15 cm diameter) and introduced in the cage. The males were allowed to feed on the ME (hereafter called ME-Fed males) for 1 h (09:30–10:30 h; peak ME foraging time, Wee and Tan, 2000). The feeding activity of individual males was not monitored during exposure period of 1 h. The petri-dish containing the ME was then removed, and the treated filter paper strip was sealed in a polythene bag and discarded. The males were provided with standard adult diet and water *ad libitum*.

2.2.2. ME-aromatherapy

ME-aromatherapy was performed in another room isolated from the fly culture room and ME-feeding room. Marking of males as described earlier was done 1d before treatment. Marked males (15–16d old, $n = 100$) were transferred to a plexiglass tubular cage (20 cm × 64 cm diameter) having both openings covered with cloth mesh. ME (0.5 mL) was introduced in the same manner described above, except that the petri-dish was covered with fine nylon mesh that prevented male contact with the ME source. The males were exposed to ME volatiles (hereafter called ME-Aroma-Treated males) for 3 h (09:30–12:30 h). The males started to move away from the petri-dish after 3 h. and slight shaking of petri-dish resulted in all males moved away. Therefore, ME exposure for 3 h was adopted. The petri-dish was then removed, and the ME-laden paper strip was sealed in a polythene bag and discarded. The males were provided with standard adult diet and water *ad libitum*.

2.2.3. No-ME treatment

Male flies that were not exposed to any ME exposure (hereafter called untreated males) were maintained on standard adult diet and water *ad libitum* in another room isolated from rooms used for ME-feeding or ME-aromatherapy. Untreated males were marked on the same day as the treated males and maintained in cages in the same manner as the treated males. When tested, untreated males were 18–19d old.

2.3. Field cages

The field cages used for mating trials were screened, circular tents (2.2 m high × 2 m diameter, (Calkins and Webb, 1983), each containing a potted citrus tree of 2 m height. Eight such field cages were placed inside a large greenhouse (24 m × 10 m × 4 m) that allowed us to carry out eight replicates of the test simultaneously. A temperature of 26 ± 2 °C and 60 ± 5% RH was maintained through-out the experiment. The insect green house had semi-natural illumination due to a translucent roof.

2.4. Experiment 1. Mating competitiveness of ME-Fed males against untreated males

Wee et al. (2007) reported that ME-feeding enhanced the mating competitiveness of *B. carambolae* males 3d after feeding, therefore, ME-Fed males were evaluated 3d after ME exposure. Twenty ME-Fed males and 20 untreated males were released simultaneously in a field cage 90 min before sunset. Males of the congeneric species *B. cucurbitae* and *B. dorsalis* start pheromone calling approximately 90 min before sunset (Arakaki et al., 1984) and a similar window of time was therefore selected for the present trials. Fifteen minutes after male release, we introduced 20 virgin females (same age as that of males) into the field cages. As soon as mating occurred, the pairs were collected separately in each vial and coaxed. Experiments concluded when males stopped calling in complete darkness, one hour after sunset. Artificial illumination was used to collect any mating pair in the darkness. The pairs were brought to laboratory for identification of colours marked to differently treated males. After noting down the colour, pairs were left there to complete their mating. Eight replicates were evaluated simultaneously.

2.5. Experiment 2. Mating competitiveness of ME-Aroma-Treated males against untreated males

The same protocol used in experiment 1 was followed, except that in this experiment males were ME-Aroma-Treated instead of ME-Fed and twenty-four replicates were evaluated. Eight replicates were evaluated per day and repeated three times to determine whether the results are reproducible.

2.6. Experiment 3. Mating competitiveness of ME-Fed males against ME-Aroma-Treated males

Procedures were the same as in experiment 1, except that ME-Aroma-Treated males were used instead of untreated males. Eight replicates were evaluated simultaneously.

2.7. Experiment 4. Mating competitiveness of ME-Aroma-Treated males against ME-Fed males and untreated males

Procedures were similar to the preceding experiments, except that ME-Fed, ME-Aroma-Treated, and untreated males were all competing for mating with virgin females. Thus, the male:female sex ratio in a given field cage was 3:1 (60:20). Eight replicates were evaluated simultaneously.

2.8. Data analyses

Differences in relative mating success (number of matings out of total possible matings) by ME-Fed males vs. untreated males, ME-Aroma-Treated males vs. untreated males, and ME-Fed vs. ME-Aroma-Treated males fulfilled parametric assumption (data were normally distributed) and were analysed by the unpaired *t*-test. Mating differences among ME-Fed, ME-Aroma-Treated, and untreated males in experiment 4 also fulfilled parametric assumption (data were normally distributed) and were analysed by One-Way ANOVA. The significance value used in data analysis was 95% ($\infty = 0.05$). Complementary pair-wise comparisons of means were performed by Tukey's test (Ott and Longneaker, 2001).

3. Results

In a competition between ME-Fed males and untreated males (Experiment 1) the ME-Fed males achieved significantly higher ($t = 4.52$, df = 14, $P < 0.001$) mating success than untreated males (Fig. 1). Similarly, ME-Aroma-Treated males competing with untreated males (Experiment 2) achieved significantly higher ($t = 5.35$, df = 46, $P < 0.0001$) mating success than untreated males (Fig. 2). While ME-Aroma-Treated males competing with ME-Fed males (Experiment 3) showed no difference ($t = 0.91$, df = 14, $P = 0.37$) in mating success between both treated males (Fig. 3). In another comparison where ME-Fed males, ME-Aroma-Treated males, and untreated males were competing with each other (Experiment 4), mating success among the differently treated males ($F_{1,23} = 7.68$, $P < 0.001$) was significantly different. ME-Fed males and ME-Aroma-Treated males achieved similar mating success, and both were significantly higher (Tukey's Test, $P < 0.05$) than that observed for untreated males (Fig. 4).

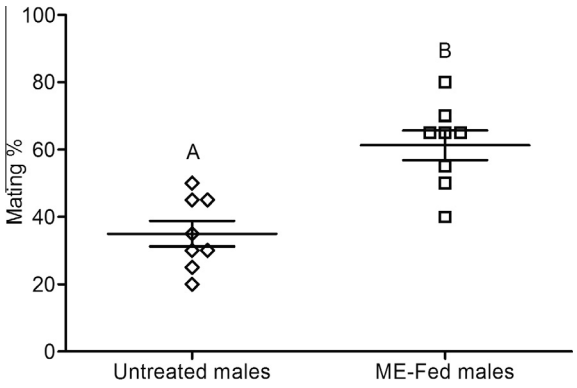

Fig. 1. Mean mating percentage of ME-Fed or untreated *Bactrocera carambolae* males. ME-Fed males ($N = 160$) were competing with 160 untreated males for 160 virgin females of same age under field cage conditions. Male age was 18d, treated with ME 3d before mating test, and eight replications were evaluated. Symbols represent raw data for 8 replicates; horizontal lines represent mean + SE. Mean male mating success followed by different letters are significantly different from each other (Student's *t*-test, $P < 0.05$).

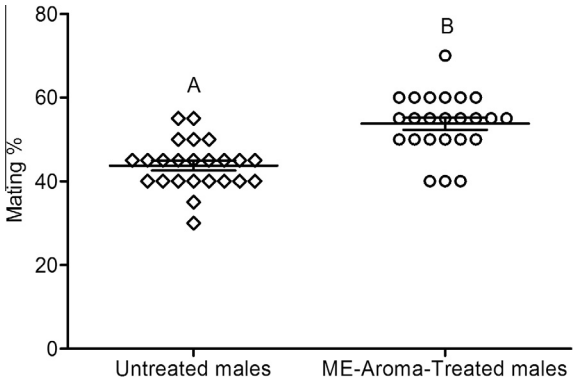

Fig. 2. Mean mating percentage of ME-Aroma-Treated or untreated *Bactrocera carambolae* males. ME-Aroma-Treated males ($N = 480$) were competing with 480 untreated males for 480 virgin females of same age under field cage conditions. Male age was 18d, treated with ME 3d before mating test, and 24 replications were evaluated. Symbols represent raw data for 24 replicates; horizontal lines represent mean + SE. Mean male mating success followed by different letters are significantly different from each other (Student's *t*-test, $P < 0.05$).

Fig. 3. Mean mating percentage of ME-Fed or ME-Aroma-Treated *Bactrocera carambolae* males. ME-Aroma-Treated males ($N = 160$) were competing with 160 ME-Fed males for 160 virgin females of same age under field cage conditions. Male age was 18d, treated with ME 3d before mating test, and eight replications were evaluated. Symbols represent raw data for 8 replicates; horizontal lines represent mean + SE. Mean male mating success followed by different letters are significantly different from each other (Student's *t*-test, $P < 0.05$).

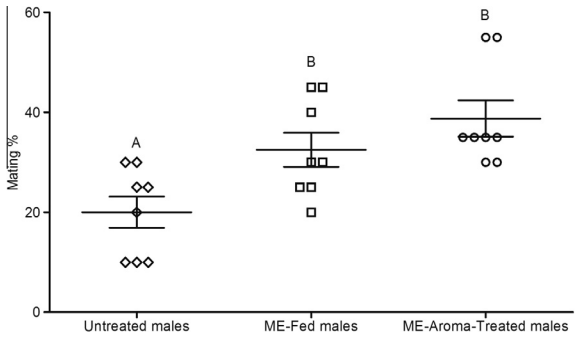

Fig. 4. Mean mating percentage of ME-Fed, ME-Aroma-Treated, or untreated *Bactrocera carambolae* males. ME-Aroma-Treated males ($N = 160$) were competing with 160 ME-Fed and 160 untreated males for 160 virgin females of same age under field cage conditions. Male age was 19d, treated with ME 3d before mating test, and eight replications were evaluated. Symbols represent raw data for 8 replicates; horizontal lines represent mean + SE. Mean male mating success followed by different letters are significantly different from each other (Tukey's Test, $P < 0.05$).

4. Discussion

Wee et al. (2007) reported that ME feeding enhances *B. carambolae* male mating competitiveness 3 days after feeding. Our results are consistent with these previous findings as ME-Fed males achieved significantly higher mating success than untreated males. Similarly application of ME-aromatherapy also increased the mating success 3 days after exposure over untreated males. Mating success of ME-Aroma-Treated males was similar to that of ME-Fed males, and both male groups were significantly better at mating with virgin females than untreated males. Although the mating success of ME-Aroma-Treated males was significantly higher than that of untreated males, the difference in mating success was smaller in comparison to that of ME-Fed males over untreated males.

ME is an active compound naturally found in many plant species (Metcalf and Metcalf, 1992; Tan and Nishida, 2012). *B. dorsalis* and *Bactrocera papayae* males convert the ingested lure into two main components, 2-allyl-4,5-dimethoxyphenol (DMP) and trans-coniferyl alcohol (CF), with trace amounts of cis-3,4-dimethoxycinnamyl alcohol (DCA) (Nishida et al., 1988a, 1988b; Tan and Nishida, 1996). In *B. carambolae*, the majority of ME is apparently converted to CF (Wee et al., 2007), and both metabolites of ME (DMP and CF) are stored in the rectal glands (Nishida et al., 1988b; Wee et al., 2007). These metabolites have also been detected in the male pheromone, i.e. DMP in the pheromones of *B. dorsalis* and *B. papayae* (Nishida et al., 2000, 1988b) and CF in the pheromone of *B. carambolae* (Wee et al., 2007). Furthermore, females have been shown to be attracted to the ME derivatives presented either singly or in a blend (Hee and Tan, 1998; Khoo et al., 2000; Nishida et al., 1997; Tan and Nishida, 1996). Based upon these findings it has been assumed that only feeding can provide sufficient ME to enhance male mating success.

The testing of an alternative way of offering ME such as inhalation/aromatherapy was the result of a casual observation. During an evaluation of the effects of ME on male mating behaviour of different species of the *B. dorsalis* complex, we noticed that *B. carambolae* responded more slowly to ME sources compared to *B. dorsalis* s.s. and *Bactrocera invadens* (unpublished data). Previous studies had also shown that *B. carambolae* responded seventeen and nine times less to ME than *B. dorsalis* and *B. papaya*, respectively (Wee et al., 2002). Data collected in the field in Suriname also indicated that a lower number of *B. carambolae* were attracted to ME baits and four to five times more ME fibre blocks were needed per hectare to obtain similar suppression as compared to *B. dorsalis* (van Sauers-Müller, 2008). Based upon these

observations, it was assumed that *B. carambolae* responded differently to ME than *B. dorsalis*. When we introduced an ME source inside the cage, most *B. dorsalis* males immediately rushed to the ME source, but *B. carambolae* males approached the ME-source more slowly and remained at some distance away from the ME-source. Nevertheless these *B. carambolae* males were apparently processing the volatiles, actively pumping with the proboscis and being affected by appearing sluggish after inhalation of the ME vapours. This casual observation led to the hypothesis that *B. carambolae* males may be capable of utilizing ME volatiles rather than requiring direct feeding on the chemical.

When a petri-dish covered with fine netting that prevented contact with the ME was introduced in a cage, the *B. carambolae* males rushed instantly to the source, and all males congregated on the mesh at the top of the petri-dish. Wee et al. (2002) demonstrated that *B. caramabolae* males were the least sensitive to ME among sibling species *B. papayae* and *B. dorsalis* and higher doses of ME were required to sensitize *B. carmbolae* compared to the ME dose required to sensitize *B. papayae* or *B. dorsalis* males. In the ME-aromatherapy set up, the petri-dish covered with fine netting may have accumulated the ME volatiles beneath the net and *B. carambolae* males may have been able to detect these accumulated volatiles of ME more quickly.

If *B. carambolae* males need to feed on ME and then to metabolize it for pheromone production, the question begs itself of how the aromatherapy can have a similar effect on male mating success as ME feeding. Studies on pharmacophagy of ME showed that minute amounts of ME (0.01 μl) were sufficient to achieve the desired mating effect in *B. caramabolae* (Wee et al., 2007). It seems that in aromatherapy experimental set up males possibly were able to take up the desired amount of ME in the form of volatiles by pumping with their proboscis or, alternatively, the ME may have been absorbed through the cuticle while sitting on top of the screened petri dish. If this is the case of ME volatiles intake either pumped in through proboscis or absorbed through cuticle, ME should be bio transformed to pheromonal components and released in pheromones. These studies suggested that chemical composition of pheromones of ME-Aroma-Treated males and ME-Fed males should be done to more fully evaluate the behavioural results reported herein.

Despite the finding that ME feeding enhances male *B. carambolae* mating success, there has been no effective way of providing the sterile males in an emergency and release facility with this chemical. In analogy with the ginger root oil aromatherapy that is routinely being practised in Mediterranean fruit fly emergence and release facilities in the world, providing the sterile males with enough ME through aromatherapy opens new avenues to enhance the mating competiveness of these flies and hence the effectiveness of the SIT for this pest species. Enhanced mating success due to ME exposure will reduce the numbers of sterile males that need to be released, which will reduce the cost of SIT applications (McInnis et al., 2011). If ME application by aromatherapy could also prevent sterile males from responding to ME sources in the field, this would allow applying simultaneously both MAT and SIT resulting in a synergetic control strategy that could increase dramatically control efficiency and cost reduction (Barclay et al., 2014).

Acknowledgements

We are deeply thankful to Thilakasiri Dammalage, Sohel Ahmad, and Ulysses St. Tomas for their tireless assistance in rearing the flies and field cage tests.

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